Characterization of mitochondrial FOXRED1 in the assembly of respiratory chain complex I


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Abstract

Human mitochondrial complex I is the largest enzyme of the respiratory chain and is composed of 44 different subunits. Complex I subunits are encoded by both nuclear and mitochondrial (mt) DNA and their assembly requires a number of additional proteins. FAD-dependent oxidoreductase domain-containing protein 1 (FOXRED1) was recently identified as a putative assembly factor and FOXRED1 mutations in patients cause complex I deficiency; however, its role in assembly is unknown. Here, we demonstrate that FOXRED1 is involved in mid-late stages of complex I assembly. In a patient with FOXRED1 mutations, the levels of mature complex I were markedly decreased, and a smaller ∼475 kDa subcomplex was detected. In the absence of FOXRED1, mtDNA-encoded complex I subunits are still translated and transiently assembled into a late stage ∼815 kDa intermediate; but instead of transitioning further to the mature complex I, the intermediate breaks down to an ∼475 kDa complex. As the patient cells contained residual assembled complex I, we disrupted the FOXRED1 gene in HEK293T cells through TALEN-mediated gene editing. Cells lacking FOXRED1 had ∼10% complex I levels, reduced complex I activity, and were unable to grow on galactose media. Interestingly, overexpression of FOXRED1 containing the patient mutations was able to rescue complex I assembly. In addition, FOXRED1 was found to co-immunoprecipitate with a number of complex I subunits. Our studies reveal that FOXRED1 is a crucial component in the productive assembly of complex I and that mutations in FOXRED1 leading to partial loss of function cause defects in complex I biogenesis.

Introduction

Mitochondrial complex I (NADH:ubiquinone oxidoreductase) is the largest complex of the mitochondrial respiratory chain (1). It is located in the mitochondrial inner membrane and protrudes into the matrix to form an L-shaped structure (2) and also assembles with other respiratory chain complexes to form respirasomes (3). Isolated defects in complex I are the most common of the mitochondrial respiratory chain disorders (4,5) and complex I dysfunction has also been implicated in Parkinson’s disease (6,7) and aging (8). In humans, complex I consists of 44

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different subunits (9–11), with one subunit located at two separate sites (2). Seven subunits, ND1–ND6 and ND4L, are encoded by mitochondrial DNA (mtDNA) and are found in the mitochondrial arm or proton translocation module (P module). The remaining 37 subunits are encoded by nuclear DNA (nDNA) and imported into the mitochondria (12–14). Seven of the nDNA-encoded subunits (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7, and NDUFS8) are ‘core subunits’ of the peripheral, or matrix, arm of complex I, catalyzing the oxidation of NADH (at the N module) and electron transfer (at the Q module) to ubiquinone (15,16). The remaining 30 nDNA-encoded subunits are referred to as ‘accessory’ subunits, and are proposed to assist in complex I biogenesis and support the structural stability of the enzyme (9,12,17). The recent structural characterization of the bovine enzyme supports this concept (2).

Complex I assembly is proposed to involve a stepwise process with intermediate complexes shared by two assembly pathways: de novo synthesis headed by mtDNA-encoded subunits and the dynamic exchange of newly imported nDNA-encoded subunits with pre-existing components of the mature complex (18–20). The complicated assembly of such a large number of subunits into the mature holocomplex I requires a number of assembly factors that are not part of the final structure of complex I. To date, at least 12 known or putative complex I assembly factors have been described and mutations in 9 of these are associated with isolated complex I deficiency due to impaired complex I biogenesis (19,21–23).

While defects in assembly cause mitochondrial disease, it was recently reported that efficient assembly of complex I is also associated with longevity in mice (8). It is therefore important to understand the intricacies of the assembly pathway to determine potential therapeutic approaches to combat disease and aging. Phylogenetic profiling and subsequent knockdown studies identified FOXRED1 as a candidate protein for complex I biogenesis (24). Two separate studies subsequently found that mutations in FOXRED1 cause complex I deficiency and mitochondrial disease (25,26). However, it is thus far not known how FOXRED1 functions in complex I biogenesis. Here, we demonstrate that FOXRED1 acts as a crucial factor in the productive assembly of complex I intermediates and that patient mutations represent partial loss of function that can be overcome by overexpression.

Results

FOXRED1 is associated with the matrix face of the mitochondrial inner membrane

FOXRED1 is a 54 kDa protein with a putative FAD-binding site and lacking a predicted transmembrane anchor. It was suggested that FOXRED1 may contain a cleavable mitochondrial targeting signal (26), but this has not been biochemically addressed. In vitro import analysis was conducted where radiolabeled FOXRED1 was incubated with isolated mitochondria for various times, and import monitored by SDS–PAGE (27). FOXRED1 migrated at its predicted size of 54 kDa (Fig. 1A, lane 1). After incubation with mitochondria for various times, the size of FOXRED1 did not alter, although it was protected from externally added proteinase K, indicating that like a subset of matrix-directed proteins (28,29), FOXRED1 lacks a cleavable targeting signal. Furthermore, like other proteins imported into the inner membrane or matrix (30), FOXRED1 import was dependent on the presence of a mitochondrial membrane potential (ΔΨm, Fig. 1A, lane 9).

To confirm the sub-mitochondrial location of FOXRED1, we expressed a C-terminal FLAG-tagged construct of FOXRED1 in HEK293T cells. Mitochondria were isolated and incubated in a low-sucrose concentration buffer to induce outer membrane rupture, followed by treatment with exogenous proteinase K (27). FOXRED1 was detected as a protease-resistant protein, suggesting that it was facing the mitochondrial matrix (Fig. 1B, lane 4). As controls, the outer membrane protein Tom20 was degraded by protease treatment both before and after outer membrane rupture (Fig. 1B, lanes 2 and 4), while Tim23, an inner membrane

Figure 1. Mitochondrial localization of FOXRED1. (A) Radiolabeled FOXRED1 was incubated for various times (10–60 min) with mitochondria isolated from HEK293T cells in the presence or absence of a membrane potential (ΔΨm). Samples were treated with or without proteinase K (Prot. K) and were subjected to SDS–PAGE and phosphorimage analysis. (B) Mitochondria were isolated from HEK293T cells expressing FOXRED1FLAG, followed by outer membrane rupture (swelling) and treatment with Prot. K. Samples were subjected to SDS–PAGE and western blot analysis. The proteins Tim23 and Tom20 served as controls for an outer membrane protein facing the intermembrane space and an outer membrane protein, respectively. The protein NDUFS2 was used as a control for matrix localization. (C) Mitochondria were isolated from HEK293T cells expressing FOXRED1FLAG and subjected to sonication or sodium carbonate (Na2CO3) treatment. After ultracentrifugation, supernatant (S) and pellet (P) fractions were subjected to SDS–PAGE and western blot analysis. Total indicates the mitochondria without any treatment. Antibodies against NDUFB6 and NDUFS2 and NDUFA1 were used as peripheral membrane proteins and cytochrome c (Cyt. c) as a soluble intermembrane space protein.
protein that faces the intermembrane space of the inner membrane, was initially protected from protease treatment (Fig. 1B, lane 2), but was degraded following outer membrane rupture (Fig. 1B, lane 4). Additionally, the complex I subunit NDUFS2, which is located in the mitochondrial matrix, was protected from protease treatment after outer membrane rupture (Fig. 1B, lane 4). In order to determine whether FOXRED1 associates with the inner membrane, mitochondria were isolated from HEK293T cells transfected with the FOXRED1FLAG construct and either sonicated or subjected to alkaline extraction with sodium carbonate (27). FOXRED1 was found in the pellet but not the supernatant fraction after sonication, indicating its membrane association (Fig. 1C, lanes 2 and 3). After alkaline extraction, FOXRED1 was found in the supernatant but not the pellet (Fig. 1C, lanes 4 and 5), suggesting that it peripherally associates with the inner membrane. The membrane arm complex I subunit NDUFB6 and the matrix arm complex I subunit NDUFS2 were used as controls for integral and peripheral membrane proteins, respectively. The complex I assembly factor, NDUFA1 (CIA30), which peripherally associates with the inner membrane (31), was also detected in the pellet after sonication and carbonate extraction (Fig. 1C, lanes 3 and 5) while cytochrome c was found in the soluble fraction (Fig. 1C, lanes 2 and 4). The combined import and immunoblot results indicate that FOXRED1 resides on the matrix face of the inner membrane, consistent with its role in complex I biogenesis.

**FOXRED1 patient cells have reduced complex I and accumulate an ~475 kDa subcomplex that is rescued upon complementation**

Compound heterozygous mutations in the FOXRED1 gene were identified in a patient with Leigh syndrome showing isolated complex I deficiency (25). The two mutations in the FOXRED1 gene were a c.694C > T transition resulting in a Gln232 to premature stop alteration (p.Q232*) and a 1289A > G transition resulting in an Asn430 to Ser change (p.N430S).

As we have found significant variability in oxygen consumption between different control fibroblasts, robust differences in respiration measurements compared with patient cells is not always observed. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were thus determined using FOXRED1 patient fibroblasts and four different control fibroblast cell lines. As can be seen (Fig. 2A), the ECAR/OCR ratio was greater in FOXRED1 patient cells, suggesting that these cells were more reliant on glycolysis. Next we analyzed the steady-state levels of individual respiratory chain holocomplexes by solubilizing mitochondria from control and FOXRED1 patient fibroblasts in the detergent Triton X-100 (TX), followed by BN-PAGE and western blot analysis. The amount of mature complex I in FOXRED1 patient mitochondria was strongly reduced compared with that of control fibroblasts, whereas the levels of complexes II, III and IV appeared normal (Fig. 2B). These results are consistent with the diagnosis of 9% residual activity of complex I in patient fibroblasts (25). Furthermore, nDNA-encoded complex I subunits NDUFS5, NDUFB6, NDUFS2, NDUFS3 and the mtDNA-encoded ND1 were decreased in patient fibroblast mitochondria compared with the control (Fig. 2C).

To investigate the assembly of complex I subunits in FOXRED1 patient mitochondria, we performed 2-dimensional BN-PAGE analysis (2D-PAGE) followed by western blot analysis using specific antibodies. All subunits tested were present in the mature complex; however, their amounts were strongly decreased in the patient mitochondria compared with the control (Fig. 2D). Interestingly, the nDNA-encoded subunit NDUFS5 and the mtDNA-encoded subunit ND1 were also observed in an ~550 kDa complex in the control. This complex represents the previously reported ~650 kDa subcomplex (19), but we now use the more accurate re-estimations of subcomplex sizes performed by Andrews and colleagues (32). In FOXRED1 patient mitochondria, these subunits were predominantly detected in a smaller complex which we refer to as being ~475 kDa. NDUFS5 has not previously been found in any detectable assembly intermediate and is thought to assemble into the holocomplex at a late stage, although a recent study suggested its presence in the ~815 kDa (previously ~830 kDa) complex that represents the mature complex lacking addition of the N-module (33). Further investigations are required to determine the entry point of this intermembrane space subunit in the CI assembly pathway. Using NDUFB6 antibodies, we detected the ~550 kDa subcomplex in both control and patient mitochondria, although the signal was weaker in the patient. In contrast to NDUFS5 and ND1, NDUFB6 was not found in the ~475 kDa subcomplex but was present in a lower form in the patient cell mitochondria. NDUFB6 is thought to assemble into an ~370 kDa (formerly ~460 kDa) intermediate along with subunits including ND2, ND3 and ND4L, although defects in assembly, including cells deficient in ND5 or ND6 lead to the appearance of NDUFB6 in smaller subcomplexes (34). NDUFS2 was also detected in lower molecular-weight subcomplexes in patient mitochondria, and similar complexes have been observed in previous studies. For example, NDUFS2 has been found in early complex I intermediates including an ~200 kDa soluble subcomplex and a 315 kDa membrane subcomplex (formerly the 400 kDa intermediate) (20). In addition, it has recently been shown that NDUFS2 is methylated at Arg85 by the assembly factor NDUFA7 (35,36) and this stabilizes formation of the ~315 kDa intermediate that contains additional subunits including ND1. As ND1 is predominantly found at the ~475 kDa subcomplex in FOXRED1 patient cells, the NDUFS2 subcomplexes most likely represent early soluble species. This suggests that the loss of FOXRED1 likely slows the assembly process leading to the accumulation of stalled intermediates.

In order to verify that the reduced level and assembly defects of complex I in FOXRED1 patient cells were indeed caused by a FOXRED1 deficiency, complementation analysis was performed. Expression of FOXRED1 in lentiviral transduced patient fibroblasts resulted in increased levels of mature complex I (Fig. 2E, compare lanes 2 and 3) with concomitant loss of the ~475 kDa subcomplex. In contrast, transduction of the lentiviral vector lacking FOXRED1 had no effect on complex I levels (Fig. 2E, lane 4). We conclude that the mutations in FOXRED1 lead to reduced complex I assembly.

**FOXRED1 is involved in the assembly/stability of late stage complex I intermediates**

More precise assessment of complex I assembly defects can be monitored using pulse-chase assembly analysis of mtDNA-encoded subunits (37,38). Control and FOXRED1 patient fibroblasts were pulse labeled with [35S]-Met, followed by a chase of up to 24 h to enable unlabeled subunits to assemble with the radiolabeled subunits. Mitochondria were then isolated and subjected to 2D-PAGE (Fig. 3A). In control fibroblasts, the signals of mtDNA-encoded complex I subunits were observed in a characteristic ~815 kDa complex at 0 h chase (39), and after 24 h were all found in mature complex I (Fig. 3A, bottom left panel). However, while in FOXRED1 patient fibroblasts the mtDNA-encoded
subunits assembled into the ∼815 kDa complex following labeling (0 h chase), they did not subsequently assemble into the mature complex and were detected in a smaller ∼475 kDa complex (Fig. 3A, right).

The complex I assembly factor NDUFAF1 (CIA30), along with other assembly factors, associates with complex I intermediates, ranging from ∼315 to 815 kDa (22,31,40). To characterize the ∼475 kDa complexes in FOXRED1 patient mitochondria, we performed antibody shift assays with NDUFAF1 antibodies. After mtDNA-encoded subunit labeling, mitochondrial lysates were incubated with NDUFAF1 or pre-immune antibodies prior to SDS-PAGE (41). In order to differentiate shifted complex I subunits from other mtDNA-encoded proteins, the lane was excised and subjected to SDS-PAGE in the second dimension (Fig. 3B). In both control and FOXRED1 patient mitochondria, complex I subunits ND1 and ND2 in the ∼815 kDa were shifted with NDUFAF1 antibodies (Fig. 3B, top panels), indicating that the ∼815 kDa complex in the FOXRED1 patient mitochondria associates with NDUFAF1, like that of control fibroblast mitochondria. However, at 6 h chase where ND1 and ND2 were detected in the ∼475 kDa complex in FOXRED1 patient mitochondria, NDUFAF1 antibodies were unable to shift these subunits (Fig. 3B, bottom panels). The dissociation of NDUFAF1 suggests that the ∼475 kDa complex in FOXRED1 patient fibroblasts is not a stalled assembly intermediate but rather it represents a stable but crippled subcomplex due to defects in the late stage assembly pathway of complex I.

**FOXRED1 gene disruption leads to severe complex I deficiency**

Our work indicates that mutations in FOXRED1 lead to defects in the assembly of complex I. Notably, some complex I is still able to
be assembled and this may explain the reason for the patients surviving into adulthood (25,26). The mutations in FOXRED1 may therefore represent a partial loss of function. To address whether FOXRED1 is absolutely necessary for complex I assembly, we used TALEN-mediated gene editing of HEK293T cells and analyzed two cell lines where expression of FOXRED1 has been disrupted (the first clone is denoted ΔFOXRED1 and the second clone, ΔFOXRED1C2). We targeted the first coding exon and identified nucleotide deletions in both clones, all of which resulted in loss of the translation initiation codon (Fig. 4A). Proteomic analysis of mitochondria isolated from these cells revealed the absence of FOXRED1 peptides in comparison to control mitochondria (data not shown). In addition, both ΔFOXRED1 clones were unable to grow on media containing galactose as the sole carbon source with ~80% cell death observed after 72 h (Fig. 4B). Similar growth defects were also previously reported following gene disruption of NDUFA9 in HEK293T cells that arrested complex I assembly (33).

Western blotting with complex I subunit antibodies revealed a minor amount of assembled complex I in both the holoenzyme form (following TX solubilization) and supercomplex form (following digitonin solubilization) (Fig. 4C and D). In addition, ΔFOXRED1 mitochondria harbored NDUFS5 in an ~475 kDa complex similar to FOXRED1 patient cells.

Given their similarity, subsequent analysis focused on ΔFOXRED1 cells. Analysis of select steady-state levels of complex I subunits revealed that NDUFS2, NDUFS3 and NDUFV2 were strongly reduced in ΔFOXRED1 mitochondria, while the levels of subunit NDUFB6 appeared unchanged (Fig. 4E). The levels of NDUFA9, a subunit required for joining the membrane and matrix arms (33) and intermembrane space localized NDUFS5 were slightly reduced. In addition, the relative levels of select complex I assembly factors were unaltered (Fig. 4E), suggesting that the defect in complex I assembly is not due to indirect effects related to the biogenesis of these proteins. BN-PAGE analysis of mitochondria isolated from ΔFOXRED1 cells also revealed the absence of complex I in-gel activity (IGA; Fig. 4F, left panel) while respiratory complexes II, III and IV showed that the holoenzyme forms were largely unaltered. Enzyme measurements revealed that ΔFOXRED1 and ΔFOXRED1C2 clones displayed 13 and 11% complex I activity respectively, relative to control cells. The presence of complex III and complex IV in complex I-containing supercomplexes was severely disrupted in ΔFOXRED1 mitochondria, as expected (Fig. 4F).

ΔFOXRED1 cells exhibit a strong respiratory defect

In order to investigate the effect of the loss of FOXRED1 on respiration, we undertook analysis of OCRs and ECARs. The basal ECAR/OCR ratio was greater in ΔFOXRED1 cells, indicating an increased reliance on glycolysis for energy production in this cell
Figure 4. Generation of a ΔFOXRED1 cell line with a specific complex I defect. (A) Nucleotide sequence spanning the TALEN-targeted region in the FOXRED1 gene present in the first coding exon. Sequence analyses of corresponding alleles in ΔFOXRED1 and ΔFOXRED1<sup>112</sup> cells indicate a deletion of 6–16 nucleotides encompassing the initiation codon. (B) Control and ΔFOXRED1 clones were plated on glucose or galactose-containing media, incubated for 72 h and analyzed for cell viability by trypan blue staining (>300 cells/assay; n = 3, SD). (C) Mitochondria isolated from control, ΔFOXRED1 and ΔFOXRED1<sup>112</sup> clones were solubilized in Digitonin (Dig) or TX before BN-PAGE followed by immunodecoration with the antibodies against complex I subunits NDUFA9 and NDUFS5. (D) Mitochondria isolated from control and ΔFOXRED1 cells were subjected to BN-PAGE as in (C) followed by immunodecoration with the antibodies against complex I subunits NDUFS2, NDUFS3 and NDUFB6. (E) Mitochondria from control and ΔFOXRED1 cells (10–40 µg protein) were subjected to SDS-PAGE and western blot analysis using antibodies against complex I subunits, assembly factors and control proteins, as indicated. (F) Mitochondria from control and ΔFOXRED1 cells were subjected to BN-PAGE followed by an IGA assay for complex I or western blot analysis using antibodies for complexes II (SDHA), III (Core I) and IV (COXIV).
Further analysis of the OCR in ΔFOXRED1 cells revealed a reduced basal respiration rate, a reduced oligomycin-sensitive respiration rate due to membrane proton leak and a reduced maximal respiration rate relative to control cells (Fig. 5B). In the presence of the drugs rotenone and antimycin A, which inhibit complexes I and III, respectively, oxygen consumption was comparable, indicating no significant difference in non-mitochondrial oxygen consumption (Fig. 5B). In addition, the OCRs of both ΔFOXRED1 and ΔFOXRED1C2 cell lines were indistinguishable (Fig. 5C). We conclude that while FOXRED1 is not essential for assembly, it is nevertheless critical for ensuring efficient complex I assembly to levels that sustain oxidative phosphorylation.

Overexpression of FOXRED1-containing pathogenic mutations can rescue assembly

Rescue studies were undertaken in order to confirm that the loss of complex I in ΔFOXRED1 cells was due to a lack of FOXRED1 protein. Transient expression of FOXRED1 fused to either a FLAG-epitope tag or GFP (both at the C-terminal end) was able to rescue the defect in complex I assembly (Fig. 6A). In addition, no defects in complex I assembly were seen following FOXRED1 overexpression in control HEK293T cells.

Two separate missense mutations have been reported in FOXRED1 that lead to complex I dysfunction—p.R352W (26) and p.N430S (25). Each mutation disrupts a highly conserved residue (Fig. 6B). Interestingly, overexpression of either FOXRED1R352W or FOXRED1N430S was able to rescue complex I assembly in ΔFOXRED1 cells (Fig. 6C, upper panel). Analysis by SDS–PAGE and western blotting using anti-FLAG antibodies confirmed similar expression of FOXRED1 forms (Fig. 6C, lower panel). Transfected cells were also able to efficiently grow on galactose-containing media, indicating that mitochondrial respiration was restored (data not shown). These results suggest that the mutations are hypomorphic in nature.

Figure 5. ΔFOXRED1 cells have a strong respiratory defect. (A) Measurement of the basal ECAR: basal OCR ratio for control and ΔFOXRED1 cells. (N = 3, SEM). (B) The OCR of control and ΔFOXRED1 cells was measured over a 170 min period. The addition of oligomycin was used to measure proton leak, while the maximal respiration was measured by addition of the membrane potential uncoupler FCCP. Non-mitochondrial respiration was measured by addition of rotenone and antimycin A, which inhibit CI and CIII, respectively. N = 3, SEM. (C) Representative traces of OCRs for control, ΔFOXRED1 and ΔFOXRED1C2 cells as described in (B). Error bars represent the SEM of 6–7 replicates/sample.
Figure 6. Complementation analysis of ΔFOXRED1 cells. (A) Control or ΔFOXRED1 cells expressing FOXRED1FLAG or FOXRED1GFP were subjected to BN-PAGE and immunoblotting with NDUFA9 antibodies to detect complex I. SDS-PAGE analysis and western blotting using FLAG and GFP antibodies was used to confirm expression of the fusion proteins. Tom20 was used as a loading control. (B) Partial sequence alignment of FOXRED1 from various species. The position of the patient mutations p.R352W and p.N430S is indicated. (C) Mitochondria isolated from control or ΔFOXRED1 cells transiently expressing FLAG-tagged FOXRED1 or patient mutations, p.R352W and p.N430S were subjected to BN-PAGE and immunoblot analysis for complex I (NDUFA9; top panel). Expression was confirmed using SDS-PAGE and immunoblot analysis with FLAG antibodies, while Tom20 was used as a loading control. (D) Mitochondria isolated from control or ΔFOXRED1 cells transiently expressing FLAG-tagged FOXRED1 variants were subjected to BN- and SDS-PAGE and immunoblot analysis as described in (C).
residues to investigate their role in FOXRED1 function and possible FAD binding. Tyrosine, cysteine and histidine residues are capable of being covalently flavinated, while tyrosine and phenylalanine are also capable of non-covalent stabilization of FAD through interactions with the phenyl moiety (42-44). Residues Y327, Y349 and Y359 were chosen based on high conservation within FOXRED1 in different species. Also, the predicted structural similarity of FOXRED1 with sarcosine oxidase (MSOX) (26) led us to select residues Y410 and Y411, as these two tyrosine residues align closely with the site C316, the site of covalent attachment of FAD in MSOX (45). While mutations in residues Y327, Y349, Y410 and Y411 were able to restore complex I levels to that of wild-type cells, the mutation Y359A was not (Fig. 6D, lane 9). As the Y359F mutation was still functional (Fig. 6D, lane 8), it suggests that the phenyl moiety at position 359 is critical for the function of FOXRED1 in the biogenesis of complex I. Equal levels of expression were confirmed by SDS–PAGE and western blot analysis (Fig. 6D, bottom panel).

**FOXRED1 co-immunoprecipitates with a subset of complex I subunits**

Given the lack of specific antibodies for FOXRED1, we were unable to determine whether the endogenous protein forms stable complexes. Furthermore, following ectopic expression of epitope-tagged FOXRED1 in control or ΔFOXRED1 cells, we were unable to detect the presence of specific FOXRED1-containing complexes on BN-PAGE (data not shown). FOXRED1 may therefore transiently or loosely associate with complex I intermediates which are refractory to BN-PAGE. To investigate FOXRED1 interactions, we performed co-immunoprecipitation analysis of FOXRED1-FLAG expressed in ΔFOXRED1 cells. In this case, we employed stable-isotope labeling of cells (SILAC) and subsequent quantitative mass spectrometry of eluted proteins (46,47). To correct for potential bias due reduced levels of complex I subunits in ΔFOXRED1 mitochondria (Fig. 4), we also expressed FOXRED1 lacking the C-terminal FLAG epitope in ΔFOXRED1 cells (see Fig. 7A for more details). In addition to this, we also performed co-immunoprecipitation analysis of the inactive FOXRED1<sup>Y359A</sup>-FLAG mutant in the same manner to determine proteins specifically bound to the functional FOXRED1 (Fig 7A). As can be seen (Fig. 7B and C, Supplementary material, Tables S1 and S2), FOXRED1, but not the mutant FOXRED1<sup>Y359A</sup>, co-immunoprecipitated with a number of complex I subunits including NDUFB10, import or biogenesis) while contaminating proteins (A) bind non-specifically to the resin in all cases. Upon washing and elution, FOXRED1-containing complexes are enriched in FLAG-tagged elutions while contaminating proteins elute in all samples. Following SILAC analysis, contaminating proteins are removed, while true FOXRED1-binding proteins are enriched. (B) Mitochondria from heavy or light amino acid-labeled ΔFOXRED1 cells expressing FOXRED1 or FOXRED1<sup>ΔCAT</sup> were subjected to immunoprecipitation with anti-FLAG beads. Elutions were mixed and analyzed by LC–MS. The means of normalized heavy/light ratios (log<sub>10</sub>), identified in at least two replicates (including a label switch; N = 4) were plotted against their P-values (−log<sub>10</sub>). Thresholds were set at P-values < 0.05 and mean heavy/light enrichment ratios >6. For simplicity, only bona fide mitochondrial proteins present in the MitoCarta dataset are shown, and commonly used protein names are substituted for gene names (Hsp10, HSPE1, Hsp60, HSFD1, mt-Hsp70, HSPA9, Miro-2, HKT2; MPPalpha, MPPbeta, MPPgamma, Tim23, TIMM8A; Tim13, TIMM13; Tim20, TOMM20; Tom22, TOMM22). Unfiltered data can be found in Supplementary material, Table S1. (Q) Mitochondria from heavy or light amino acid-labeled HEK293T cells expressing FOXRED1<sup>Y359A</sup> or FLAG-tagged FOXRED1<sup>Y359A</sup> were subjected to anti-FLAG immunoprecipitation and analysis performed as in (A). Unfiltered data can be found in Supplementary material, Table S2.

![Figure 7. FOXRED1-FLAG co-immunoprecipitates with a subset of complex I subunits](https://academic.oup.com/hmg/article-abstract/24/10/2952/623776/2960)

(A) Schematic representation of FOXRED1 co-immunoprecipitation approach. ΔFOXRED1 cells were transfected with a construct encoding wild-type or inactive mutant p.Y359A FOXRED1, with and without a FLAG tag. Expression of FOXRED1 is able to restore levels of complex I subunits in both the tagged and untagged samples. FOXRED1 (denoted X) is able to bind to interacting partners (Y represents a protein required for import or biogenesis) while contaminating proteins (Δ) bind non-specifically to the resin in all cases. Upon washing and elution, FOXRED1-containing complexes are enriched in FLAG-tagged elutions while contaminating proteins elute in all samples. Following SILAC analysis, contaminating proteins are removed, while true FOXRED1-binding proteins are enriched.
complex I intermediates into respirasomes (48). and are likely to occur at stages in assembly prior to assembly of which stabilizes the assembly in the absence of N-module subunits (33). Indeed, the unproductive component FOXRED1 leads to instability and breakdown of the assembly factors including NDUFAF1, Ecsit, ACAD9 and other breakdown products of the observations have been seen in cells lacking the NDUFA9 subunit hydrophobic arm was largely intact. The reasons for such in- Not all mtDNA-encoded complex I subunits, indicating that the subcomplexes lacking NDUFA11 (32). Together, these data suggest that FOXRED1 may function in a complex comprising at least the core subunit NDUFS3 and the accessory subunits NDUFA5, NDUFA10, NDUFB10 and NDUFS5. Proteomic analysis failure to detect any subunits of other respiratory complexes, sug- gesting FOXRED1 performs its function prior to incorporation of complex I intermediates with other respiratory complexes. Assembly of complex I with other complexes has been suggested to occur following assembly of the ~815 kDa subcomplex, and before assembly into holocomplex I (39,48). Because we are unable to identify subunits belonging to other respiratory complexes, we can conclude that FOXRED1 most likely exerts its function prior to this stage, thus being a mid-stage assembly factor for the biogenesis of complex I.

**FOXRED1 patient mutations remain partially functional**

Two separate missense mutations in FOXRED1, resulting in the substitutions p.R352W (26) and p.N430S (25), lead to loss of complex I activity and mitochondrial disease. Most patients with nuclear gene defects and complex I dysfunction display severe symptoms and do not survive to adulthood (21). Interestingly, both patients harboring FOXRED1 mutations survived to adulthood. This indicates that the mutations result in partial loss of function and/or loss of FOXRED1 can be compensated by other factors. Our studies indicate that the former is most likely true since total loss of FOXRED1 leads to severe reduction in complex I levels while overexpression of either of the mutant forms in these cells restores complex I. Thus, the pathogenic mutations in FOXRED1 are most likely hypomorphic in nature. Similar observations have also been for the complex I assembly factor NUBPL where overexpression of a p.Gly56Arg missense mutation could overcome a complex I defect in patient cell lines (50). It is therefore possible that future therapeutic treatments could involve mechanisms to increase the synthesis of these mutant proteins so that the reduced assembly activity is dampened. Current therapies for complex I deficiency and mitochondrial disease include supplementation with various metabolites such as dichloroacetate, CoQ10, creatine or riboflavin (51,52). While symptoms may improve for some patients, it has been shown that responsiveness may depend on the particular gene mutation present, leading to a non-responsive phenotype (53,54). Our data suggest that future therapies could include the identification of pharmacological agents that enhance expression of the mutant protein, thereby overcoming effects of pathological mutations and restoring levels of complex I.

**Discussion**

Loss of FOXRED1 leads to impaired complex I assembly and the presence of a crippled subassembly While it has been established that FOXRED1 is involved in complex I biogenesis (24) and mutations in its gene cause mitochondrial disease related to complex I deficiency (25,26), little information is available regarding its functional role. Here we found that FOXRED1 associates with the matrix side of the inner membrane, following import of the protein in a membrane potential-dependent manner, and interacts with a subset of complex I subunits. Moreover, loss of FOXRED1 leads to defects in the latter stages of complex I assembly, resulting in the accumulation of a crippled ~475 kDa subcomplex. We believe that this ~475 kDa subcomplex represents a non-productive breakdown of a stalled assembly intermediate for the following reasons. First, pulse-chase assembly analysis indicated that at early time points, FOXRED1 patient cell mitochondria could form a late stage ~815 kDa complex I intermediate, but this was unproductive, and was followed by the formation of the ~475 kDa complex. Confirmation that this was the ~815 kDa intermediate was demonstrated by its association with the assembly factor NDUFAF1. In addition, the ~475 kDa complex was no longer associated with NDUFAF1. Secondly, the ~475 kDa complex contained most, if not all, mtDNA-encoded complex I subunits, indicating that the hydrophobic arm was largely intact. The reasons for such instability of the ~815 kDa intermediate are not clear, but similar observations have been seen in cells lacking the NDUFA9 subunit (32). Sits at the interface between the matrix and membrane arms of complex I (33). Indeed, the unproductive complex observed in ΔNDUFA9 mitochondria also contained the late assembly subunit NDUFS5, suggesting the unproductive complex to also be a breakdown product of the ~815 kDa complex.

The late stage ~815 kDa complex also interacts with various assembly factors including NDUFAF1, Ecsit, ACAD9 and other proteins that form the MCIA complex (23), as well as NDUFA2, which stabilizes the assembly in the absence of N-module subunits (49). Given the involvement of multiple assembly factors converging at this point, and our observation that loss of FOXRED1 leads to instability and breakdown of the ~815 kDa species, this suggests that the late stages of complex I assembly represent a critical nexus in the enzyme’s biogenesis.

**FOXRED1 is found in association with complex I subunits**

The exact molecular role played by FOXRED1 still remains unclear, but this is also the case for many complex I assembly factors that have been identified in the past decade (19). FOXRED1 has putative oxidoreductase activity and has homology to FAD-binding proteins that are involved in redox reactions related to amino acid catabolism (dimethylglycine dehydrogenase, sarcosine dehydrogenase, 1-pipeolic acid oxidase and peroxisomal sarcosine oxidase) and metabolic regulation (pyruvate dehydrogenase regulatory subunit) (25,26). However, there is still no direct evidence for FAD-binding or oxidoreductase activity. Mutations of residues involved in potential FAD binding did not result in inhibition in FOXRED1 activity, except for the substitution of Tyr359 for Ala. It remains to be determined whether this mutation leads to potential defects in FAD binding, or if it causes other defects, such as impairing protein folding. Nevertheless, this construct served as a useful control to determine the proteins that can form a complex with FOXRED1.

The recent determination of a 5 Å cryo-EM structure of bovine complex I revealed not only conservation of the core subunits from T. thermophilus but also the presence of density attributed to the accessory subunits (2). This structure revealed that the subunits identified as co-immunoprecipitating with FOXRED1 (NDUFS3, NDUFA10 and NDUFS5) are located in close proximity to each other and to the mitochondrial inner membrane, consistent with the sub-mitochondrial localization of FOXRED1. Furthermore, HA-tagged NDUFS5 has been found in association with FOXRED1 (23), while FOXRED1 was also identified in stalled complex I subcomplexes lacking NDUFA11 (32). Together, these data suggest that FOXRED1 may function in a complex comprising at least the core subunit NDUFS3 and the accessory subunits NDUFA5, NDUFA10, NDUFB10 and NDUFS5. Proteomic analysis failed to detect any subunits of other respiratory complexes, suggesting FOXRED1 performs its function prior to incorporation of complex I intermediates with other respiratory complexes. Assembly of complex I with other complexes has been suggested to occur following assembly of the ~815 kDa subcomplex, and before assembly into holocomplex I (39,48). Because we are unable to identify subunits belonging to other respiratory complexes, we can conclude that FOXRED1 most likely exerts its function prior to this stage, thus being a mid-stage assembly factor for the biogenesis of complex I.
Materials and Methods
Cloning and molecular biology

The FOXRED1 open reading frame was amplified from a cDNA library and cloned into the pGEM4Z vector (Promega, Madison, WI, USA) for import studies and also in frame with a C-terminal FLAG epitope containing pCDNA3 vector for expression studies. Site-directed mutagenesis was performed using the Phusion mutagenesis method (New England Biolabs) according to manufacturers’ instructions.

For TALEN construction, the 5’ UTR and first exon of human FOXRED1 (NM_017547) was used as the input sequence for designing suitable TALEN-biding pairs using the ZifIT Targeter Version 4.2 (16). The binding pair FOXRED1-L (NG HD NI NN NI NN NN NG HD HD NN NN NN HD NG HD) and FOXRED1-R (NG NN HD HD NN NG ND HD NN HD NI NN NI NI HD HD) were, respectively, assembled into the JDS70 (NI) and JDS71 (HD) backbones, yielding pTALEN-FOXRED1-L and pTALEN-FOXRED1-R, according to Reyon et al. (55).

Cell culture

Patient and control fibroblasts used in this study have been reported previously (25). Generation and genomic verification of the ΔFOXRED1 cell lines was performed according to Stroud et al. (33). Isogenic HEK293T cells were used as a control. Cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin with 50 µg/ml uridine. Cells were grown at 37°C under an atmosphere of 5% CO2. For cell viability measurements, cells were grown in glucose or galactose-containing media for 72 h before harvesting adherent and non-adherent cells and staining with 0.2% trypan blue. Cells (>300/sample) were subsequently counted.

For SILAC, cells were cultured in SILAC DMEM (Thermo-Fisher Scientific; 4.5 g/l glucose, 4 mM l-glutamine, 110 mg/l sodium pyruvate) containing 10% (v/v) dialyzed FBS, supplemented with penicillin/streptomycin, 50 µg/ml uridine, 600 ng/ml L-proline and either ‘light’ amino acids (146 mg/l l-lysine-HCl and 42 mg/l l-arginine-HCl) or ‘heavy’ amino acids (180 mg/l 15N4-l-lysine and 44 mg/l 15N4-l-arginine; Cambridge Isotope Laboratories). All cells were cultured at 37°C under 5% CO2, 95% air.

Complementation studies

For the lentiviral-mediated rescue of patient fibroblasts, the FOXRED1 open reading frame was cloned into the pGEM4Z vector (Promega) and then subcloned into the 4-hydroxytamoxifen-inducible lentivector particle pF-5X-UCMS-SV40-puroGa-HER26VP16 (GEV16)-W as described in (31). Lentiviral particles were produced and infected into cells as previously described (31).

Mitochondrial localization analysis

Mitochondria were isolated according to the methods of Johnston et al (41). For subfractionation, mitochondria were resuspended in either 10 mM MOPS (pH 7.2) and 250 mM sucrose or 10 mM MOPS (pH 7.2), and treated with 0.1 mg/ml proteinase K for 30 min on ice (27). Alternatively, isolated mitochondria were subjected to either sonication in 100 mM NaCl and 10 mM Tris–HCl (pH 7.6) or alkaline extraction in freshly prepared 0.1 M Na2CO3 (pH 11.5). Membranes were pelleted at 100 000g for 30 min at 4°C, and supernatants were precipitated with trichloroacetic acid (27). After treatments, soluble (supernatant) and insoluble (pellet) fractions were subjected to SDS–PAGE and western blot analysis.

Radiolabeling studies

mtDNA-encoded translation products were labeled using previously described methods (56). For import studies, radiolabeled FOXRED1 was translated using TnT Coupled Reticulocyte Lysate (Promega) in the presence of a [35S]-methionine/cysteine. Translated protein was incubated with mitochondria isolated from HEK293T cells at 37°C for various times. Proteinase K treatment and dissipation of membrane potential were performed as described elsewhere (39).

Co-immunoprecipitation analysis

For immunoprecipitation, SILAC-labeled ΔFOXRED1 cells were transfected with pCDNA3 plasmid harboring DNA encoding FOXRED1–FLAG, FOXRED1Δ55A–FLAG or FOXRED1Δ35A and allowed to express protein for 48 h. Mitochondria were then isolated and solubilized in lysis buffer (20 mM Bis-Tris, pH 7, 50 mM NaCl, 10% glycerol) containing 1% (w/v) digitonin. Individual lysates were incubated with anti-FLAG M2 affinity gel (Sigma) for 2 h at 4°C with gentle rotation. Following washing with lysis buffer containing 0.1% (w/v) digitonin, bound protein was eluted with 100 µg/ml FLAG peptide (Sigma).

Mass spectrometry and data analysis

Proteins were acetone precipitated, solubilized in 8 M urea, 0.05% ProteaseMax (Promega), 50 mM ammonium bicarbonate, and reduced and alkylated with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride and 50 mM iodoacetamide at 37°C for 30 min. Proteins were digested with LysC (Promega) in the presence of a [35S]-methionine/cysteine. Translation products were labeled using previously described methods (39). Peptides were analyzed by an online nano-HPLC/electrospray ionization-MS/MS on an LTQ-Orbitrap Elite Instrument connected to an Ultimate 3000 HPLC (Thermo-Fisher Scientific). Peptides reconstituted in 0.1% TFA and the peptides desalted on SDB-XC (Empore) StageTips as previously described (57). Peptides were analyzed by an online nano-HPLC/electrospray ionization-MS/MS on an LTQ-Orbitrap Elite Instrument connected to an Ultimate 3000 HPLC (Thermo-Fisher Scientific). Peptides reconstituted in 0.1% TFA and 2% acetonitrile (ACN) were loaded onto a trap column (C18 PepMap 100 µm ID × 2 cm trapping column, Thermo-Fisher Scientific) at 30 °C under acidic conditions (0.1% TFA and 2% acetonitrile). Peptides were then separated by a reversed-phase column (C18, 3 µm, 300 Å and 75 µm ID × 25 cm trapping column, Thermo-Fisher Scientific) at 5 µl/min for 6 min, and washed for 6 min before switching the precolumn in line with the analytical column (Vydac MS C18; 3 µm, 300 Å and 75 µm ID × 25 cm, Grace Pty. Ltd.). The separation of peptides was performed at 300 nl/min using a non-linear ACN gradient of buffer A (0.1% formic acid, 2% ACN) and buffer B (0.1% formic acid, 80% ACN), starting at 5% buffer B to 55% over 120 min. Data were collected in Data-Dependent Acquisition mode using m/z 350–1500 as MS scan range, CID for MS/MS of the 20 most intense ions. Other instrument parameters were: MS scan at 120 000 resolution, maximum injection time 150 ms, AGC target 1E5, CID at 35% energy for a maximum injection time of 150 ms with AGC target of 5000.
For data analysis, Thermo MS raw files were analyzed using the MaxQuant platform (version 1.5.0.30); (58) searching against the human UniProt FASTA database (release 38 July 2014; 88993 entries) and a database containing common contaminants by the Andromeda search engine (59). Default setting parameters were used with modifications. Briefly, cysteine carbamidomethylation was used as a fixed modification, and N-terminal acetylation and methionine oxidation were used as variable modifications. False discovery rates of 1% for proteins and peptides were applied by searching a reverse database, and ‘Re-quantify’ and ‘Match between runs’ options were enabled. Unique and razor peptides were used for quantification, using a minimum ratio count of 1. Data analysis was performed using the Perseus software. SILAC ratios (heavy/light) were filtered for proteins quantified by >1 unique peptide and >5% sequence coverage. Mean normalized ratios were calculated from each of these proteins identified in >2/4 replicates and log2 transformed. p-values across replicates were calculated by a one-tailed t-test. Gene names were matched to gene symbols and synonyms listed in the human MitoCarta dataset (24).

**Oxygen consumption and enzyme activity measurements**

OCR and ECARs were measured in live cells using a Seahorse Biosciences XF24-3 Analyzer according to manufacturer’s procedures. Briefly, 50,000 HEK293T cells were plated per well of a poly-α-lysine-treated Seahorse Biosciences culture plate and grown overnight in standard culture conditions. OCR and ECAR were analyzed in cells in non-buffered media with the following inhibitors: 2 µM oligomycin, 0.5 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.3 µM antimycin A along with 0.5 µM rotenone. Four measurement cycles (2 min mix, 2 min wait, 5 min measure) were done for basal conditions, and following each inhibitor injection. For each cell line, 7–10 replicates were measured in triplicate plates and measurements normalized to cell number with CyQuant (Life Technologies). Mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration. To calculate proton leak and mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration were normalized to cell number with CyQuant (Life Technologies). Mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration. To calculate proton leak and mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration were normalized to cell number with CyQuant (Life Technologies). Mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration. To calculate proton leak and mitochondrial OCR was determined from basal OCR minus non-mitochondrial respiration were normalized to cell number with CyQuant (Life Technologies).

**SDS–PAGE, BN-PAGE and western blot analysis**

SDS–PAGE was performed using Tris–Tricine gradient gels (61) and BN-PAGE and second dimension SDS–PAGE was performed according to Lazarou et al. (39). BN-PAGE molecular-weight markers employed were thyroglobulin (669 kDa), ferritin (440 kDa) and BSA (137 and 67 kDa) and were employed as reference markers rather than true size estimations. In-gel activity assays were performed according to Zerbetto et al. (62). Antibodies were purchased for NDUFS2, NDUFS3, SDHA, Core I and COXIV (Sapphire Biosciences), COII (Invitrogen), NDUFV2 and FLAG-epitope (Sigma), Tom20 (Santa Cruz, CA, USA), Tim23 and cytochrome c (BD Transduction Laboratories), while rabbit polyclonal GFP, NDUFA9, NDUFB6, NDUFS5, NDUFAF1, NDUFAF2, NDUFAF4, Ecsit, mtHsp70 and ACADE were made in-house. The ND1 antibody was a gift from A. Lombès (Paris, France). Secondary probing with α-mouse or α-rabbit horseradish-peroxidase-conjugated antibodies (Sigma) was performed for 2 h, followed by detection using Enhanced Chemiluminescence reagents (GE Healthcare, NJ, USA) and a ChemiDoc Imaging system (Bio-Rad, Hercules, CA, USA).

**Supplementary Material**

Supplementary Material is available at HMG online.

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